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Rubella Virus Capsid Protein Induces Apoptosis in Transfected RK13 Cells

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Rubella virus is an enveloped positive-strand RNA virus that can cause mild to severe birth defects or death in an infected fetus. RV induction of programmed cell death, demonstrated in cell culture, has been implicated in the pathogenesis. The timing of apoptosis, 48 h p.i., suggested that accumulation of RV structural proteins might induce cell death in infected cells. Expression of RV structural proteins, capsid, envelope glycoproteins E1 and E2, in transiently transfected RK13 cells was as potent an inducer of cell death as RV infection. Immunofluorescence microscopy revealed that RV structural protein transfected cells exhibited the condensed nuclei typical of apoptotic cell death. Transfection with the capsid protein construct, but not E2 and E1, resulted in as much cell death as joint expression of all three RV structural proteins. Capsid required a membrane-anchoring domain to induce cell death, but a heterologous polypeptide fused to the capsid membrane anchor did not cause apoptosis. Deletion mutants demonstrated that the apoptosis-inducing activity resides in the N-terminal 170 amino acids of capsid. Though apoptosis-inducing capsid constructs appear to have an ER sub-cellular localization, disruption of the ER calcium storage capacity does not correlate with cell death. Mechanisms consistent with these results are discussed.

INTRODUCTION

Rubella virus (RV) is a positive-strand, RNA virus of the family Togaviridae. The most severe pathogenic effect of RV, congenital rubella syndrome (CRS), occurs when a woman is infected in the first trimester of gestation. This syndrome results in alteration in fetal organ development (Wolinsky, 1996) and may be caused by the virus's ability to induce apoptosis (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999; Pugachev and Frey, 1998).

Induction of apoptosis by virus infection is emerging as an important aspect of pathogenesis. Therefore uncovering the mechanism of the initiation of apoptosis is an intense area of research (reviewed in Roulston *et al.,* 1999). The diversity of proposed mechanisms suggests that each virus engages the apoptotic machinery according to the virus's particular physiology. In some cases, specific viral proteins have been identified that are potent apoptosis inducers by themselves such as adenovirus E1a (Rao *et al.,* 1992) or chicken anemia virus apoptin (Noteborn *et al.,* 1994). In other cases, the presence of viral RNA, rather than viral proteins, is implicated as a trigger. Examples of this are the apoptotic action of the RNA-dependent 2–5A synthetase/RNase L system (Castelli *et al.,* 1997) and the RNA-dependent protein kinase, PKR (Lee and Esteban, 1994; Lee *et al.,* 1997). Among positive-strand RNA viruses, several mechanisms for apoptosis have been described. Semliki Forest virus requires the nonstructural proteins for induction of apoptosis (Glasgow *et al.,* 1998). Sindbis virus requires a membrane fusion event that does not require RNA replication (Jan and Griffin, 1999) and may be mediated by the transmembrane domains of the envelope glycoproteins (Joe *et al.,* 1998). Therefore, a detailed understanding of each virus life cycle must be combined with detailed knowledge of the cellular response to viral infection to explain the mechanisms of apoptosis induction.

The RV infection cycle is initiated when the capped, polyadenylated plus-sense genome is uncoated in the cytoplasm and translated to synthesize the two nonstructural proteins (reviewed in Frey, 1994). These viral proteins, possibly in association with host proteins (Atreya *et al.,* 1998; Duncan and Nakhasi, 1997; Nakhasi *et al.,* 1988, 1994), catalyze the replication of a complementary copy of the genome. This negative strand is used as a template to replicate the genomic RNA and to transcribe a 24S subgenomic messenger RNA. The 24S RNA is translated into a polyprotein that contains capsid and two envelope glycoproteins, E2 and E1. A signal peptide between capsid and E2 guides the synthesis to the surface of the endoplasmic reticulum (ER) and remains attached to capsid when E2 is cleaved by the signal peptidase, thereby anchoring the majority of capsid in

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membranes. The majority of the capsid polypeptide chain remains oriented toward the cytoplasm (Suomalainen *et al.,* 1990). E2 and E1 are type I membrane proteins that are processed and form heterodimers in the ER (Hobman *et al.,* 1993). All three structural proteins are transported to the Golgi (Hobman, 1993) where virus particles assemble as they bud in most cell types without the appearance of preformed nucleocapsids (Garbutt *et al.,* 1999; Wolinsky, 1996). This ordered process from translation to assembly takes place in the absence of genomic RNA if structural proteins are coordinately expressed in cells (Hobman *et al.,* 1994).

Previous studies have demonstrated that the cytopathology due to RV infection results from apoptosis, though the precise mechanism that initiates the cell-death program is not clear (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999; Pugachev and Frey, 1998). The timing of apoptosis induction, which is delayed ≥48 h after cells are infected with RV at an m.o.i. of 5 (Duncan *et al.,* 1999), may provide a clue. At 48 h p.i., viral replication and shedding into the media has occurred (Frey, 1994). The delay suggests that accumulation of virus components late in the cycle may be required for induction of cell death. To determine whether the RV proteins alone could induce apoptosis, in the current study we expressed RV structural proteins from a plasmid that mimics the later stages of infection in transfected cells. Results show that the expression of capsid protein containing a membrane-anchoring domain is required to cause cell death at a level similar to RV infection.

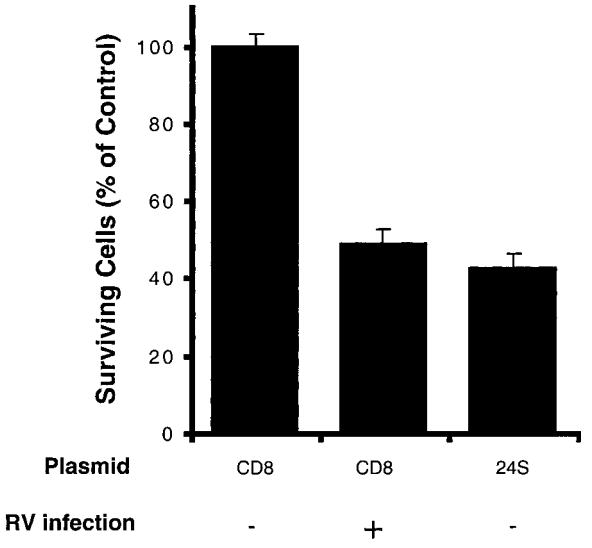

FIG. 1. Expression of RV structural proteins causes cell death at the same level as virus infection. The graph shows the proportion of live, GFP-expressing cells relative to the mean number of surviving, mock-infected cells transfected with CD8. Cell counts were made 48 h after transfection with the construct indicated under each bar. The CD8 construct expresses the human cell surface protein, CD8. The 24S construct expresses the three RV structural proteins, capsid, E2, and E1. Cells were RV- or mock-infected immediately after transfection. Constructs are described in Fig. 4. Each column represents the mean of six independent transfections with error bars of one standard error of the mean.

RESULTS

Rubella virus structural proteins induce apoptosis

Previous studies showed that RV replication was required for apoptosis induction (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999). However, the delay of 48 h before substantial cell death, as well as the absence of substantial cell death in cell lines that only support a low level of RV infection (Frey, 1994), suggested that the accumulation of viral components was necessary (Duncan *et al.,* 1999). RV structural proteins accumulate with these kinetics (Frey, 1994). To investigate the effect of RV structural proteins in the absence of viral replication, RK13 rabbit kidney cells were transiently transfected with expression plasmids encoding RV structural proteins. This cell line was chosen because it is very sensitive to RV-induced apoptosis (Duncan *et al.,* 1999). Cells were cotransfected with a plasmid that encodes the RV structural proteins, pCMV5–24S (Hobman *et al.,* 1990), and a plasmid encoding the green fluorescent protein (GFP). The expression of GFP allowed identification of cells in culture that had taken up plasmid DNA. The number of live green cells after transfection with the 24S plasmid was compared to the number of live green cells similarly cotransfected with a control protein, human T cell surface protein, CD8. When the green fluorescence was first visible (~12 h), the number of green cells on the RV construct transfected plates and the CD8 transfected plate were similar, indicating similar transfection efficiency (~20%). In parallel, CD8/GFP-cotransfected cells were infected with RV at an m.o.i. of 5, and the number of surviving, green cells, 48 h p.i. was counted. Similar counts were made for uninfected, CD8/GFP-cotransfected cells and 24S/GFP-cotransfected cells at 48 h posttransfection. The number of surviving, transfected (green) cells on each plate was divided by the mean number of green cells on the CD8 control transfected plates. This value showed a 48.9% mean survival for RV-infected cells (Fig. 1), corresponding to the amount of RV-induced cell death previously observed for nontransfected cells (Duncan *et al.,* 1999). The 42.5% survival of RV construct transfected cells reflects cell death at about the same level as that caused by RV infection (Fig. 1). Although every GFP-expressing cell may not be infected with RV or be expressing from the 24S plasmid, the percent of cell survival relative to the control reveals the lethal effect of RV or the structural proteins alone.

To demonstrate that the 24S transfected cells were dying by apoptosis, transfected cells were stained with the blue fluorescent, Hoechst dye that freely penetrates cells and binds to DNA, thereby revealing nuclear mor-

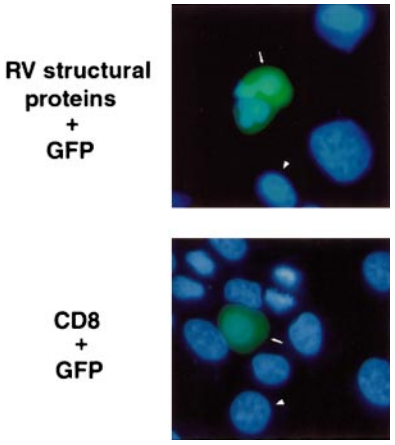

FIG. 2. RV structural protein transfected cells have condensed nuclei typical of apoptotic cells. Fluorescent micrographs of RK13 cells transfected with the GFP and RV structural protein expression constructs (top) or GFP and CD8 (bottom). Nuclei of all cells are stained with Hoechst dye, transfected cells have green cytoplasm. The arrows indicate transfected cells, the arrowheads indicate normal nuclei in adjacent, nontransfected cells.

phology. Some of the GFP-expressing cells that were cotransfected with the 24S cDNA construct showed condensed nuclei typical of apoptotic cells (Fig. 2). In contrast, cells transfected with the control plasmid that encodes CD8 retained the normal rounded nuclei as observed in neighboring, nontransfected cells.

To rule out the possibility that the apoptotic effect of RV structural proteins might be due to their expression far in excess of that seen in RV-infected cells, we transfected cells with differing amounts of the 24S plasmid and compared protein expression and cell survival to the levels in RV-infected cells. The level of capsid, a nonglycosylated protein that resolves as a single band on a Western blot, provided the clearest measure to compare the expression of structural proteins in 24S-transfected cells to the level expressed in RV-infected cells. Cell lysates were collected 48 h post-RV infection when there were ~50% of cells surviving relative to the control. Lysates were also collected from plates transfected with a range of concentrations of the 24S plasmid after counting live green cells. Each transfection contained 100 ng of the GFP plasmid and a compensating amount of an empty vector plasmid to ensure consistent transfection efficiency (~20% of cells). Lysates were separated on SDS–PAGE, and the immunoblot was developed with a polyclonal antibody to recombinant RV capsid protein (Fig. 3A). At 10 ng of transfected plasmid, the amount of capsid expressed and the level of apoptosis induced parallel the levels of capsid and cell death seen in RV-infected cells. When the amount of 24S plasmid transfected into cells was reduced, the lower level of capsid in these cells coincided with the diminution of the apoptotic effect (Fig. 3B). Thus the apoptosis observed in transfected cells correlated with the expression of RV structural proteins. These correlations suggested that apoptosis induced by transfected structural proteins might reflect the mechanism responsible for apoptosis induced by RV infection.

RV capsid induces apoptosis

Having shown that transfection with the plasmid expressing RV structural proteins in concert induced apoptosis, we sought to determine whether any of the three RV structural proteins has the same effect when transfected separately. Plasmids were constructed that express either the capsid alone or the E2–E1 polyprotein precursor. The open reading frame in the capsid construct included the E2 signal peptide. The E2–E1 construct encodes all the signals for processing of the two glycoproteins and transit out of the ER. Each of the RV structural protein expression plasmids was cotransfected with a GFP-expressing plasmid as described above. The number of live green cells was counted at

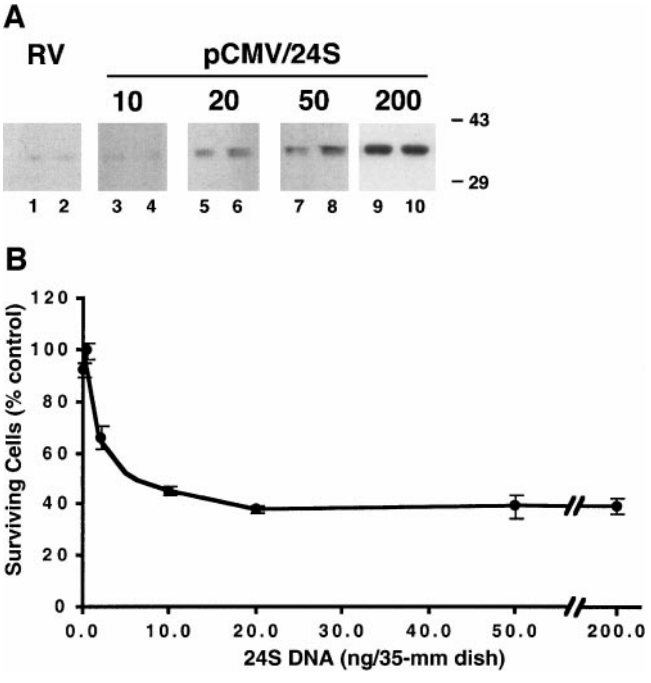

FIG. 3. Apoptosis occurs in transfected cells with capsid protein expressed at a level similar to the level required for RV-induced cell death. (A) Immunoblot of total cell lysate from RV-infected RK13 cells (lanes 1 and 2) or cells transfected with the indicated number of nanograms of the structural protein expression plasmid (pCMV/24S) per 3.5-cm dish (lanes 3–10). Thirty micrograms cell protein was loaded in each lane; the blot was developed with rabbit anti-RV capsid and visualized by chemiluminesence. The positions of molecular mass markers are indicated in kilodaltons. (B) Dose response of cell survival to amount of pCMV5/24S plasmid transfected. Mass of plasmid is given as nanograms per 3.5-cm dish of cells. The percentage of cells surviving is calculated by counting the number of green fluorescent cells on a plate transfected with pCMV5/24S and dividing by the mean number of green cells on plates transfected with pCMV5/CD8 times 100. Each point is the mean of three independent transfections. Error bars represent ± the standard error of the mean.

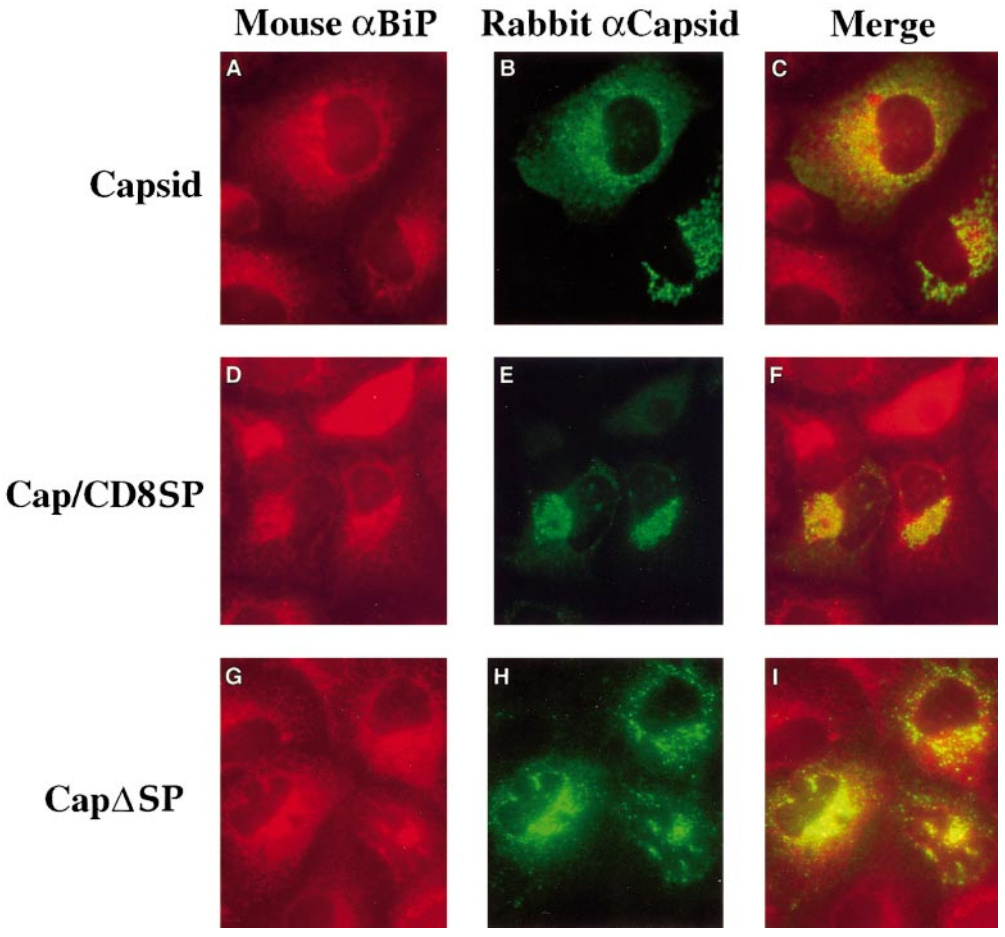

FIG. 6. Capsid constructs with membrane-anchoring domains differ in subcellular localization from capsid without the E2 signal peptide. Indirect immunofluorescence micrographs of RK13 cells transfected with plasmids expressing RV capsid (top), RV capsid/CD8 signal peptide chimera (middle, labeled Cap/CD8SP) or RV capsid terminating before the E2 signal peptide (bottom, labeled CapΔSP). Cells were double labeled with anti-BiP and anti-RV capsid antibodies. Separate images of the same field were merged to show the extent of colocalization of the expressed protein with the endogenous ER-associated BiP.

48 h and compared to cells transfected with GFP and the control protein, CD8. E2–E1 transfected cells survived at ~90% of the control cells (Fig. 4). The survival of capsid transfected cells was ~46% of the control. These results suggest that the capsid protein causes the cell death induced by RV structural proteins (42.5% survival).

Since most of the apoptotic effect of the RV structural proteins could be accounted for by the expression of the capsid, domains within the protein were investigated for their role in the apoptotic process. The importance of the E2 signal peptide membrane-anchoring domain was assessed by construction of a plasmid that encoded only the cytoplasmic domain of capsid without an E2 signal peptide (Fig. 4). The resulting capsid protein (CapsidΔSP) should not be anchored in the ER membrane surface since it lacks the E2 signal peptide. Interestingly, the number of surviving cells cotransfected with the CapsidΔSP and GFP plasmids was only 8% less than the number of cells in the control cotransfection (Fig. 4). The lack of cell death occurred despite a higher level of capsid protein expression than required for maximal cell killing when the membrane anchor was attached (data not shown).

To explore whether the E2 signal peptide itself, not the capsid protein, was responsible for cell death, two "domain swap" constructs were engineered. In the first case, the capsid open reading frame was fused to sequence encoding the CD8 signal peptide so that the capsid cytoplasmic domain should be anchored in the ER membrane using a heterologous peptide sequence. In the second case, the CD8 open reading frame from the first amino acid of the mature peptide up to the last residue prior to the *trans*-membrane domain had the sequence encoding the E2 signal peptide fused to its carboxyl terminal end. Cells were transfected with these two constructs along with the GFP plasmid, and the cell survival rates were analyzed as above. Transfection of the capsid polypeptide construct with a heterologous membrane anchor (Capsid/CD8SP) resulted in a reduced cell survival, 48% of the control, similar to the native RV protein. Yet transfection of a heterologous polypeptide anchored by the E2 signal sequence (CD8/E2SP) only

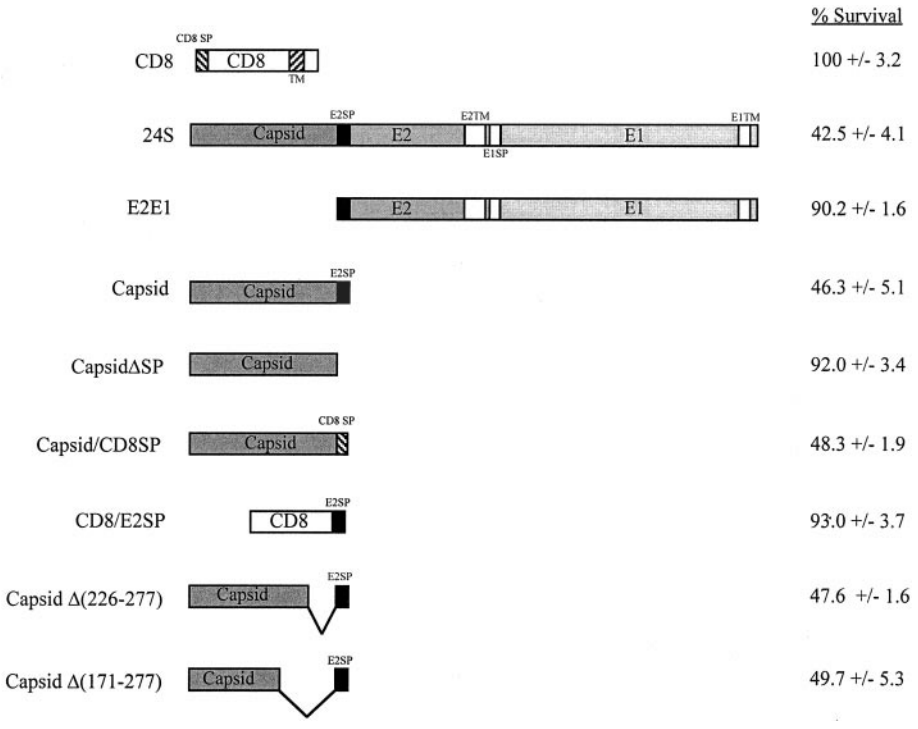

FIG. 4. Schematic of expression constructs. Horizontal boxes symbolize encoded protein sequence. The name of the cDNA construct is shown at the left. The mean percentage survival ± one standard error of the mean for at least three independent transfections shown at the right was calculated as in Figs. 1 and 3. SP, signal peptide. The numbers in parentheses in the CapsidΔ(226–277) and CapsidΔ(171–277) constructs indicate the corresponding amino acid residues deleted.

reduced survival to 93% of the control (Fig. 4). These results suggested that the primary apoptosis-inducing activity of membrane-anchored capsid resided in the cytoplasmic domain.

To further define the region within the cytoplasmic domain responsible for apoptosis, deletions were made starting from the point were the polypeptide should protrude from the membrane. Two truncation mutants were constructed in such a way that either 51 amino acids [CapsidΔ(226–277)] or 106 amino acids [CapsidΔ(171–277)] were removed from the carboxyl terminus of the capsid while leaving the truncated polypeptide still fused to the E2 signal sequence. Transfection of these two constructs resulted in a reduction in the number of surviving cells to 47.6 and 49.7% of the control, respectively, similar to the effect of the native capsid (Fig. 4). These results indicated that the apoptosis-inducing activity of capsid resides in the N-terminal 170 amino acids of its cytoplasmic domain.

Capsid-expressing cells are TUNEL positive

Though all of the above results support the role of capsid in apoptosis induction, the evidence depends on the cotransfection of an RV-capsid-expressing and a GFP-expressing plasmid. To present direct evidence of the phenotypes of RV-capsid-protein-expressing cells, flow cytometric analysis was performed. RK13 cells were transfected with plasmids (500 ng per 3.5 cm dish) that expressed capsid alone or the capsidΔSP protein that lacks the membrane-anchoring domain. Cells were harvested 48 h after transfection, fixed, permeabilized, and incubated with TUNEL reagents to incorporate fluorescein-labeled dUTP at the site of nicked DNA. Subsequently, cells were incubated with monoclonal antibody to RV capsid and a phycoerythrin-labeled second antibody. Fluorescence-activated cell scanning (FACS) and a two-dimensional dot plot of three independent transfections (a representative plot is shown in Fig. 5) revealed that capsid transfected samples had an average of eight times more capsid-positive/TUNEL-positive cells than samples transfected with the capsidΔSP construct.

A membrane anchor determines the subcellular localization of capsid

The importance of tethering the capsid polypeptide to a membrane-anchoring domain suggested that subcellular localization might be important for induction of apoptosis by this protein. To investigate the location of the various capsid-containing proteins in these transfected cells, indirect immunofluorescence microscopy was performed with a rabbit anti-capsid antibody. Cells transfected with the native capsid construct showed a distribution of antibody-reactive antigens similar, though not identical to, the ER protein, BiP (Figs. 6A–6C). The

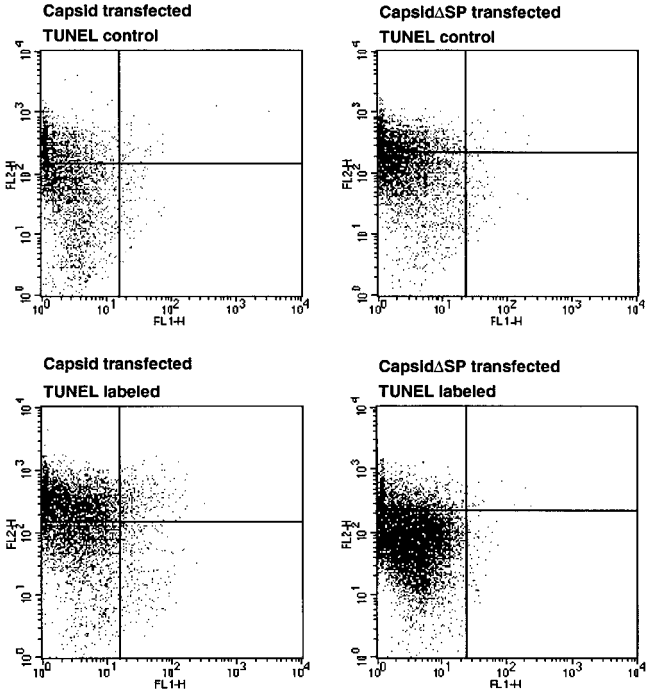

FIG. 5. The proportion of capsid expressing cells that have TUNEL labeled nuclei is eight times higher than the proportion of TUNEL-labeled cells expressing capsidΔSP as shown by dual fluorescence flow cytometry. The transfection and TUNEL labeling are indicated above these four dot plots that are representative of three independent transfections. All samples were labeled with anti-capsid antibody to identify transfected cells. The level of antibody staining is shown by the *y* axis and the cutoff for capsid positive was set by a dot plot of cells that were TUNEL labeled and incubated with the fluorescent anti-IgG antibody but not the anti-capsid antibody (not shown). The level of TUNEL staining is shown by the *x* axis, and the cutoff for positive cells was set in the upper plots so that <0.33% of total cells were positive in the TUNEL-negative control.

capsid polypeptide fused to the CD8 signal sequence (Cap/CD8SP) had a distribution similar to native capsid (Figs. 6D–6F). The localization of the nonapoptotic capsid polypeptide lacking a membrane-anchoring domain (CapΔSP) showed an intensely punctate pattern rather than the more diffuse staining seen with the other two constructs (Figs. 6G–6I). The punctate distribution of the CapΔSP construct suggested aggregates of the recombinant protein; however, the level of resolution of this methodology was too low to indicate the cell substructures with which they were associated.

Apoptosis induction did not correlate with disruption of ER calcium storage

The observation that the apoptotic effect of capsid occurred when it was anchored to the ER membrane suggested that disruption of ER function might be involved. One measure of ER function is its level of stored calcium. ER-stored calcium was measured by calculating the increase in intracellular concentration after addition of the drug thapsigargan (Lam *et al.,* 1994). Cells were perfused with medium containing EGTA to eliminate calcium influx, and the intracellular concentration was measured for 60 s after addition of the drug. Fluorescence imaging of single cells that expressed GFP allowed calculation of calcium release for ≥11 transfected cells for each construct. Among cells expressing CD8, RV structural proteins, capsid alone, or capsidΔSP, no correlation was observed between tendency to induce apoptosis and amount of calcium released (Table 1).

DISCUSSION

Discerning the trigger that initiates apoptosis in a virus-infected cell is one of the pressing goals for understanding virus pathology. The most severe pathology associated with RV, congenital rubella syndrome (CRS) (Wolinsky, 1996), may be caused, in part, by apoptotic cell death (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999; Pugachev and Frey, 1998). The results of this study suggest a possible mechanism of RV-induced apoptosis. Because each type of virus may induce apoptosis by different mechanisms, observations with types of virus that utilize similar strategies for replication could guide the interpretation of new results. Among enveloped, positive-strand RNA viruses, alphaviruses have been most extensively studied for apoptosis induction (Levine *et al.,* 1993; Scallan *et al.,* 1997). Though RV belongs to the same family as alphaviruses, studies from other family members provide little guide for interpretation of our results because contradictory mechanisms of apoptosis induction were found. For example, virus replication was required for Semliki Forest virus (SFV) (Glasgow *et al.,* 1998) but not required for Sindbis virus-induced apoptosis (Jan and Griffin, 1999). The virus proteins involved in this process have been identified as the nonstructural proteins for SFV (Glasgow *et al.,* 1998) but structural proteins for Sindbis virus (Joe *et al.,* 1998). For RV, several different mechanisms of apoptosis induction have been suggested. Active virus replication was re-

TABLE 1

ER-Stored Calcium Released after Thapsigargan Treatment of Transfected RK13 Cells.

Transfected plasmid	Cell survival (% control)	ER calcium released
CD8	100 ± 3.2	3035 ± 573
24S	42.5 ± 4.1	1715 ± 270
Capsid	46.3 ± 5.1	3051 ± 569
CapsidΔSP	92.0 ± 3.3	1679 ± 243

Note. Each of the plasmids listed in column one was cotransfected with the GFP-expressing plasmid. Surviving GFP-expressing cells are shown in column 2 as a percent of surviving CD8-transfected cells ± SE. ER calcium released is calculated by summing the intracellular calcium concentration over the 60 s after addition of the drug (nM · s), as described under Materials and Methods. Each entry is mean of 11 separate cells ± SE.

quired for RV-induced apoptosis (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999) in contrast to Sindbis-virus-induced apoptosis (Jan and Griffin, 1999). However, to achieve apoptosis in the absence of replication, cells were treated with a number of UV-inactivated Sindbis virus particles equivalent to 100 times the number required for induction of apoptosis by live virus. In RV, this large excess of UV inactivated particles has not been tested so the results can not be compared directly. The reported upregulation of the tumor suppressor p53 by RV infection (Megyeri *et al.,* 1999) confirmed in our lab for Vero cells (unpublished data) suggests the apoptotic mechanism engages the p53 pathway. However, the induction of the proapoptotic protein, Bax, (one of the downstream targets of p53) by RV infection reported by Megyeri and colleagues (1999) was contradicted by other reports (Hofmann *et al.,* 1999) and by our studies (unpublished data). Multiple studies have demonstrated the importance of caspases (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Pugachev and Frey, 1998), but this effector phase component of the apoptotic process reveals little about the inducing mechanism. Finally, strain variation in cytotoxicity that mapped to the nonstructural proteins was cited as evidence that these proteins are involved in initiation of apoptosis (Pugachev and Frey, 1998). Thus, no single hypothesis for the mechanism of RV-induced apoptosis has emerged.

The present study has demonstrated that expression of the RV structural proteins, in the absence of virus infection, induces apoptotic cell death at a rate similar to RV infection. The previously reported requirement for RV replication (Duncan *et al.,* 1999; Hofmann *et al.,* 1999; Megyeri *et al.,* 1999) may have reflected the need for viral gene expression to build up a sufficient quantity of the structural proteins to induce apoptosis by a similar mechanism. Among the structural proteins, expression of the capsid by itself can account for nearly all of the apoptotic effect. Expression of the other two structural proteins, E2 and E1, as a polyprotein did not cause substantially more cell death than the control protein. The capsid polypeptide must be fused to a membrane anchoring sequence to have the cell death effect, though the anchor may be the natural E2 signal peptide or a heterologous membrane anchoring domain. The E2 signal peptide itself does not induce apoptosis because expression of a chimeric fusion of heterologous protein sequence with the E2 signal allowed cell survival nearly at the level of the control transfection. Therefore, specific sequences in the capsid polypeptide, anchored to membrane, seem to be required for induction of apoptosis. These conclusions are supported by flow cytometric analysis that showed eight times more apoptotic cells in membrane-anchored capsid-expressing cells than in non-membrane-anchored capsidΔSP-expressing cells.

Whether the activity of capsid protein revealed in this study represents the mechanism of apoptosis induction by RV infection has not been proven. However, this activity is consistent with many of the published observations on RV-induced apoptosis. The requirement for virus replication to induce apoptosis may reflect the need to express the capsid protein. This expression will occur only after the genome is copied as a negative strand and sufficient subgenomic message is transcribed from it to direct synthesis of the structural proteins. Apoptosis induction by RV infection requires a threshold level of virus because cell lines that only support a low level of virus do not exhibit the cytopathic effect indicative of apoptosis. Apoptosis induced by capsid transfection also diminishes as the level of expressed protein drops below a critical point. Though a threshold level of capsid may be required to induce apoptosis, other factors effect the timing of apoptosis because the rate of cell survival is not affected by plasmid input in excess of 20 ng, though the amount of expressed protein is increased.

The induction of apoptosis by capsid may seem to be contradicted by reports of cell lines that stably express the three RV structural proteins (Hobman *et al.,* 1994; Hofmann *et al.,* 1999; Qiu *et al.,* 1994). In addition to the fact the RK13 has not been among the stably expressing cell lines, they have been selected for drug resistance and stable integration of the structural-protein-encoding DNA sequence. Thus these RV-protein-expressing cells, which were selected by their ability to overcome cell death, therefore could have incorporated compensating mutations that allowed the cells to tolerate the presence of capsid without being killed. The demonstration that these capsid-expressing cell lines were susceptible to chemical apoptosis inducers (Hofmann *et al.,* 1999) may only reflect different mechanisms of initiation of the process. Increased susceptibility to chemically induced apoptosis in cells persistently infected with RV was cited as evidence of different pathways of initiation of cell death (Pugachev and Frey, 1998). The transiently transfected cells in the present study were not selected for survival; therefore they reflect expression of the structural proteins in the native genetic background.

The observation that the apoptotic effect of capsid occurred when it was anchored to the ER membrane suggested that disruption of ER function might be involved. One measure of ER function is its level of stored calcium (Lam *et al.,* 1994; van Kuppeveld *et al.,* 1997). In contrast to studies where ER pool depletion correlated with cell death (Lam *et al.,* 1994), experiments where cells were transfected with the CD8, RV structural proteins, capsid alone, or capsidΔSP constructs, there was no correlation between tendency to induce apoptosis and amount of ER-stored calcium. These results suggested that capsid does not induce apoptosis by a direct disruption of the calcium storage function of the ER.

In proposing a model, capsid could initiate apoptosis by binding to a cellular protein that is linked to the apoptosis machinery. Though capsid must contain a

membrane-anchoring domain to cause apoptosis, a protein–protein interaction, rather than direct membrane disruption, is supported by the observation that the level of the ER calcium storage capacity does not correlate with apoptosis induction. The subcellular localization of the membrane-anchored capsid is similar to the location of an ER protein, BiP, and truncated capsid with an altered localization fails to induce apoptosis. This localization suggests capsid may interact with a signal cascade proposed to connect events in the ER to a nuclear apoptotic response (Urano *et al.,* 2000; Zinszner *et al.,* 1998). The capsid deletion mutants indicate that such an interaction would involve the amino terminal 170 residues because they are sufficient for apoptosis induction. Significantly, this region includes the binding site for capsid interacting protein p32 (Beatch and Hobman, 2000), a mitochondrial protein that may regulate the opening of the permeability transition pore. The opening of this pore is known to have a critical role in apoptosis (Kroemer, 1997). Further analysis of the role in the apoptotic process of this and other cellular proteins that bind to capsid is currently under way.

In conclusion, the present study showed that the membrane-anchored RV capsid can trigger cell death similar to infection by the virus. This inducing activity resides in the amino-terminal half of the cytoplasmic domain of the protein.

MATERIALS AND METHODS

Cell culture and transfection

RK13 cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.). The M33 strain of rubella virus was grown in Vero 76 cells and titered by plaque assay at 5×10^6 pfu/ml.

Cells on 35-mm culture plates were transiently cotransfected with 100 ng of the pEGFP plasmid (Clontech Laboratories, Inc.) and various amounts of an expression plasmid (described below) using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. To transfect equal amounts of DNA, pcDNA3 plasmid (Invitrogen) was included to make the total DNA in each transfection equal to 300 ng. Transfected cells were observed by direct fluorescence microscopy. Adherent cells with normal-sized nuclei were judged to be viable, and the average number of live green cells per field was determined by counting at least three fields.

Nuclear morphology was assessed 48 h after cotransfection with 100 ng of pEGFP plasmid and 100 ng of the expression plasmid per 9 cm^2 of RK13 cell monolayer on chamber slides. Hoechst 33342 (Sigma) was added to the culture medium at a concentration of 250 ng/ml for 10 min at room temperature (RT). Subsequently cells were washed with PBS and observed microscopically under PBS without fixing. Digital video images were captured at ×400 magnification first with a green then a blue filter, the two images merged with Adobe Photoshop software.

Immunoblot analysis

Protein samples were prepared as a whole cell, RIPA (1% N-P40, 0.25% Deoxycholate, 50 mM Tris, pH 7.5) lysate. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories), and 30 μg total protein was separated on 15% acrylamide SDS–PAGE as previously described (Nakhasi *et al.,* 1988). Proteins were transferred to PVDF, membranes incubated with rabbit polyclonal anti-RV capsid (Beatch and Hobman, 2000) followed by HRP-coupled donkey anti-Ig antibodies and visualized by enhanced chemiluminescence (Amersham).

Plasmid constructs

The pCMV5–24S and pCMV5-E2E1 plasmids were previously described (Hobman *et al.,* 1990). The CD8 control protein was expressed as a chimera with a cytoplasmic tail substituted with the corresponding region of the G protein of vesicular stomatitis virus (to facilitate antibody detection) from the previously described pCMV5-CD8-GTMCT (Hobman et al., *1997*). All other constructs were generated by PCR amplification of target sequences using Pfu polymerase (Stratagene). Primers contained restriction endonuclease *Eco*RI or *Hin*dIII sites for final cloning into the pCMV5 mammalian expression vector (Andersson *et al.,* 1989). The authenticity of each construct was confirmed by automated DNA sequencing.

The capsid cDNA encompasses nucleotides 6462–7406 of the rubella sequence (Dominquez *et al.,* 1990) followed by a stop codon. CapsidΔSP encompasses nucleotides 6462–7337 followed by a stop codon and encodes the capsid sequence without the E2 signal peptide. Capsid/CD8SP cDNA sequence encompasses nucleotides 6462–7337 of RV followed by the sequence encoding the CD8 signal peptide (Littman *et al.,* 1985) inserted by using a 3′ PCR primer with the sequence: 5′-*TATGATC AAGC* **TTA** CGG CCT GGC GGC GTG GAG CAG CAA GGC CAG CGG CAG GAG CAA GGC GGT CAC TGG TAA GGC GCG GAT GCG CCA AGG ATG GCG-3′. In this primer, the italicized sequence denotes a *Hin*dIII site for cloning, the bold nucleotides comprise the stop codon, the plain text encodes the CD8 signal peptide, and the underlined sequence anneals specifically to capsid cDNA template at nucleotides 7317–7337. CD8/Capsid SP cDNA was amplified from the pCMV5-CD8-GTMCT plasmid using an upstream primer that added 6 bp of the 5′ untranslated sequence of the human CD8 gene and a methionine codon to the open reading frame for the mature CD8 peptide. The final product contained the CD8 open reading frame up to codon 163 and the E2 signal peptide encoding sequence cloned in place of the

CD8 transmembrane domain. The RV E2 sequence was added using a 3′ PCR primer with the sequence: 5′-*TCGA AAGC* **TTA** GGC GCG CGC GGT GCC AAC GGC GAC CGT GGC GAG CAA GAG CCC GGC AAG GAA GGC CTG GGG GGC ACC GAA ACA GGC GAA GTC CAG CCC CCT-3′. The italicized bases denote a *Hin*dIII site, bold bases a stop codon, plain text indicates bases 7338–7406 of RV encoding the E2 signal peptide, and underlined bases anneal specifically to CD8 cDNA template just 5′ of the sequence encoding the TM domain. The CapsidΔ(226–277) cDNA encompasses nucleotide 6462–7181 of the rubella sequence by amplification with a 3′ PCR primer that adds nucleotides 7338–7406 of RV (the E2 signal peptide) and a stop codon. The CapsidΔ(171–277) cDNA encompasses nucleotide 6462–7016 of the rubella sequence by amplification with a 3′ PCR primer that adds nucleotides 7338–7406 of RV and a stop codon.

Immunofluorescence microscopy

RK13 cells were grown on fibronectin (10 μg/ml)-coated 12-mm-diameter glass coverslips. Forty-eight hours after transfection with RV expression plasmids as indicated, cells were fixed with methanol at −20°C and processed for indirect immunofluorescence using mouse anti-BiP antibody (Stresgen, Victoria, BC) and rabbit anti-capsid as described elsewhere (Hobman *et al.,* 1992).

Calcium measurement

RK13 cultures cotransfected with GFP and RV protein constructs or the CD8 control were loaded with fura-2AM solution at a concentration of 2.5 μM for 30 min and washed three times with Tyrode's buffered saline (134 mM NaCl, 2.5 mM KCl, 6 mM $NaHCO_3$, 1 mM $MgSO_4$, and 0.42 mM $NaH_2PO_4 \cdot 2H_2O$). The dishes were placed on the stage of a Zeiss Axiovert microscope at room temperature and perfused with Tyrode's buffer via a perfusion pipet (200-μm tip inside diameter) and an ALA Scientific Instruments DAD-12 superfusion system. Fluorescence of the fura-2 was excited alternately by 340 and 380 nm light from a Photon Technology Inc. (PTI) Betaram source and monochrometer. The fluorescence at 510 nm was collected with a PTI ICCD camera and Imagemaster software. The ratio of fluorescence intensity excited at the two wavelengths was used to calculate intracellular calcium in individual GFP expressing cells, using the equation given in Grynkiewicz, Poenie, and Tsien (1985). Calibration parameters were obtained from human platelets and lymphocytes by the methods previously described (Hough *et al.,* 1999). After measuring a baseline, the cells were perfused with 1 μM thapsigargin and 1 mM EGTA in nominally calcium-free Tyrode's buffer, images captured one per second for 60 s.

Flow cytometry

Forty-eight hours after transfection cells were trypsinized, washed in PBS/5% FBS, fixed in 4% paraformaldehyde and processed according to the instructions for the *In Situ* Cell Death Detection (fluorescein) Kit (Roche Molecular Biochemicals, Indianapolis, IN) for TUNEL staining. Subsequently cells were incubated 30 min on ice in permeabilization buffer (1× PBS, pH 7.4, 0.1% BSA, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM HEPES, 0.1% saponin) with 10% normal rabbit serum to reduce nonspecific binding of antibodies. After washing in PBS/5% FBS, cells were incubated 1 h on ice in permeabilization buffer with 1:100 dilution of mouse monoclonal anti-RV capsid (Chemicon International, Inc., Temecula, CA). After washing, cells were incubated with 1 μg/100μl of biotinylated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) 30 min on ice and then washed and incubated an additional 30 min with 2 μg/100μl of streptavidin-phycoerythrin conjugate (PharMingen International).

Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and CELLQuest software. Data were collected until 10,000 events were observed in the TUNEL positive range.

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